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Transcriptional upregulation of HIF-1α by NF-κB/p65 and its associations with β-catenin/p300 complexes in endometrial carcinoma cells

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The hypoxia-inducible factor (HIF)-1α, which has a major role in cell adaptation to hypoxia, is mainly regulated at post-translational levels. Recently, HIF-1α mRNA was also shown to be upregulated by several signal pathways under normoxic conditions. Here we focused on relationships of HIF-1α with NF-κB and β-catenin signaling in endometrial carcinomas (Em Cas). Long-term exposure of Ishikawa cells to cobalt chloride ($CoCl_2$), which is known to mimic the effect of hypoxia, caused a decrease in the growth, along with increased HIF-1α protein but not mRNA expression. In contrast, short-term exposure resulted in a rapid and transient increase in HIF-1α mRNA expression along with stabilization of nuclear NF-κB/p65 (p65). Transfection of p65 increased HIF-1α expression through activation of the promoter, whereas overexpression of HIF-1α also activated NF-κB-dependent transcription, indicating the existence of a positive feedback loop. In addition, HIF-1α was indirectly associated with nuclear β-catenin through interactions with p300, leading to slight enhancement of both HIF-1α- and β-catenin-mediated transcriptional activity. In clinical samples, biphasic upregulation of HIF-1α expression was observed in normal endometrial glandular components during the menstrual cycle, with the labeling indices showing significantly higher values in the early secretory stage. Significantly higher values for phosphorylated p65 and nuclear β-catenin were also observed in HIF-1α-positive than -negative lesions of Em Cas, in contrast to significantly lower Ki-67 status. These data therefore suggest that transcriptional associations with HIF-1α and NF-κB, as well as β-catenin/p300 complexes, may contribute to modulation of changes in tumor cell kinetics in response to a hypoxic condition in Em Cas.
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KEYWORDS: β-catenin; endometrial carcinoma; HIF-1α; hypoxia; NF-κB

Mammalian cells require a constant supply of oxygen to preserve adequate energy production and to maintain normal function and survival. Cellular hypoxia, characterized by a decreased O_2 tension within cells, can be caused by an environmental stress and has important implications in many pathological conditions.[1–4] For example, hypoxia promotes tumor invasion and metastasis through activation of several signaling pathways, such as Ras-extracellular signal-regulated kinase.[1] In addition, most hypoxic tumor cells are viable but non-proliferating and resemble a state of tumor dormancy, as described in non-vascularized metastatic foci.[2–4]

Hypoxia-inducible factor (HIF)-1 serves as a major transcription factor for the regulation of many genes encoding for metabolic enzymes, growth factors, extracellular matrix, and thrombosis under hypoxic condition.[5–7] In contrast to constitutively expressed HIF-1β, an inducible subunit, HIF-1α, is mainly regulated by oxygen-dependent hydroxylation of two proline residues within the O_2-dependent degradation domain. This allows the binding of the von Hipple–Lindue (VHL) tumor suppressor protein, a component of an E3 ubiquitin ligase complex that targets HIF-1α subunits for degradation by the ubiquitin–proteasome pathway.[8–12] Recently, HIF-1α mRNA has also been shown to be upregulated by several signal pathways, including NF-κB.[13–15]

In endometrial carcinomas (Em Cas), HIF-1α expression is considered to correlate with a subtype of tumor aggressiveness and activation of the classical NF-κB pathway through nuclear NF-κB/p65 accumulation.[16,17] Moreover, activation of HIF-1α signaling, as well as NF-κB, acts as a key regulator whereby tumor cells adapt to radiation therapy and hypoxia.[18] However, little is known about the functional cross-talk between HIF-1α and NF-κB signaling.

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Table 1 Primer sequences used in this study

Category	Gene		Sequence (5′–3′)	Size (bp)
mRNA	*HIF-1α*	Forward	CATGGAAGGTATTGCACTGC	365
		Reverse	TGGCAAGCATCCTGTACTGT	
	$p27^{Kip1}$	Forward	CTGCAACCGACGATTCTTCT	336
		Reverse	CATCCAACGCTTTTAGAGGC	
Promoter	*HIF-1α*	(−625)F	CTGGTAAGGAAAGACCCCGTTG	908
		(+288)R	GGTGAATCGGTCCCCGCGATG	
		kB1Mut F	AAGCC**ATTA**ACCGCCTTCCTTCGGAGA	
		kB1Mut R	GGCGGT**TAAT**GGCTTTGGGAGGCGCTGC	
		kB2Mut F	CACGAG**AATC**TTCCCGCCTCGCACCCCCA	
		kB2Mut R	GGGAA**GATT**CTCGTGAGACTAGAGAGA	
		kB3Mut F	AGCCTG**ATTA**CCGCCCGCCGTGAAGACA	
		kB3Mut R	GGCGG**TAAT**CAGGCTCGCTCCGGCCTA	
	Glut1	(−2057)F	TGGAAGTATCAGCAGGACGGACT	2075
		(+18)R	TCTCTAGCAAAATGGTGGAGCCG	
ChIP	*HIF-1α*	kB1F	CCAGGCAACCGAAATCCCTT	115
		kB1R	AGAGAAAGGAGATGGGGGTG	
		kB2F	CCCTGACAAGCCACCTGAG	122
		kB2R	GAAAGGCAAGTCCAGAGGTG	
		kB3F	CACCTCTGGACTTGCCTTTC	102
		kB3R	TCCCCGCGATGTCTTCACG	

Abbreviations: ChIP, chromatin immunoprecipitation assay; *Glut1*, glucose transporter 1; HIF-1α, hypoxia-inducible factor-1α.
Bold: 4-nucleotide alterations in the κB-binding sites.

β-Catenin is a multifunctional protein involved in E-cadherin-mediated cell–cell adhesion and acts as a downstream effector in the Wnt signaling pathway. Our previous data for Em Cas showed that β-catenin signaling may have an important role in inhibiting cell proliferation through the activation of $p14^{arf}$/p53/$p21^{waf1}$, modulating NF-κB pathways.[19–21] Given that G1 cell cycle arrest is induced by an alteration in the Myc-$p21^{waf1}$ axis under hypoxic condition,[22] it is possible that associations among HIF-1α, NF-κB, and β-catenin signaling may have an important role in modulation of tumor kinetics in response to microenvironmental hypoxia in Em Cas. To test this, extensive investigations were conducted on HIF-1α expression, together with examining NF-κB and β-catenin signaling and cell proliferation in Em Cas.

MATERIALS AND METHODS

Plasmids, Cell Lines, Antibodies, and Reagents

Full-length cDNA of human *HIF-1α* in pCMV-S-Flag vector was obtained from the Riken DNA Bank Human Resource (Tsukuba, Ibaragi, Japan). The human *HIF-1α* promoter sequence between −625 and +283, as well as the 2075-bp fragment of glucose transporter 1 (*Glut1)* promoter (NG008232), was amplified by PCR and subcloned into the pGL-3B vector (Promega, Madison, WI, USA), respectively. Site-directed mutagenesis of the putative NF-κB binding sites in the *HIF-1α* promoter was performed using the PrimeSTAR Mutagenesis Basak kit (Takara Bio, Ohtsu, Japan). In addition, a fragment of the HIF reporter, including HIF-1α regulatory elements (HREs; Qiagen, Tokyo, Japan), was subcloned into the pGL-3B vector. The identities of these constructs were confirmed by sequencing before use. The sequences of PCR primers employed in this study are listed in Table 1. pG5-luc, pcDNA3.1-mouse p65, pcDNA3.1-HA-β-catenin with deleted Serin-45 (β-catΔS45), pCI-Flag-p300, pCI-Flag-p300N, pCI-Flag-p300M, pCI-Flag-p300C, κB-luc, and Top reporters were obtained and used as described previously.[19–21,23,24]

The endometrial cancer cell lines, Ishikawa, Hec50, and Hec251 cells, as well as Ishikawa cells stably overexpressing HA-β-catΔS45, were maintained in Eagle's MEM with 10% bovine calf serum.[16–18] For hypoxia experiments, cells were treated with 100 μM cobalt chloride ($CoCl_2$) under 5% CO_2 at 37 °C.[25–27]

Anti-HIF-1α, anti-$p27^{kip1}$, anti-VHL, and anti-NF-κB/p65 were from BD Bioscience (San Jose, CA, USA). Anti-cyclin D1, anti-$p21^{waf1}$, anti-p53, and Ki-67 were from Dako (Copenhagen, Denmark). Anti-phospho-p65 at Ser275

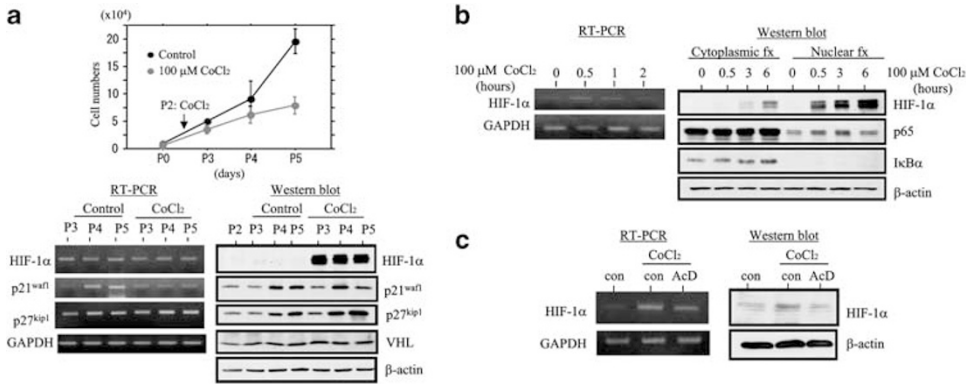

Figure 1 Hypoxia and endometrial carcinoma (Em Ca) cell kinetics. (**a**) Upper: Ishikawa cells were seeded at low density with or without 100 μM cobalt chloride ($CoCl_2$) treatment. The cell numbers are presented as means ± s.d. P0, P3, P4, and P5 are 0, 3, 4, and 5 days after cell passage, respectively. Lower: Reverse-transcriptase PCR (RT-PCR; left) and western blot (right) analyses of hypoxia-inducible factor (HIF)-1α, $p21^{waf1}$, $p27^{kip1}$, and von Hipple–Lindue (VHL) expressions in Ishikawa cells at different days of cell growth in the presence or absence of 100 μM $CoCl_2$. P2, P3, P4, and P5 are 2, 3, 4, and 5 days after cell passage, respectively. (**b**) Left: RT-PCR analysis of HIF-1α mRNA expression in Ishikawa cells with100 μM $CoCl_2$. Right: western blot analysis of HIF-1α, p65, and IκBα expression in cytoplasmic and nuclear fractions of Ishikawa cells after $CoCl_2$ exposure; fx, fraction. (**c**) Ishikawa cells were pretreated with 5 μM actinomycin D (AcD) for 2 h and exposed to 100 μM $CoCl_2$ for 1 h. HIF-1α mRNA and protein levels were determined by RT-PCR (left) and western blot (right) analyses; con, control.

(pp65), anti-phospho-Akt at Ser473 (pAkt), anti-Akt, anti-GSK-3β, anti-phospho-GSK-3β at Ser9 (pGSK-3β), and anti-IκBα were from Cell Signaling (Danvers, MA, USA). Anti-β-actin and anti-Flag were from Sigma Chemical (St Louis, MO, USA), and anti-Glut1 was from Millipore (Billerica, MA, USA). Anti-HA and anti-p300 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Transfection

Transfection was performed using LipofectAMINE PLUS (Invitrogen, Carlsbad, CA, USA), in duplicate or triplicate. Luciferase activity was assayed as described previously.[19–21]

Reverse Transcription-PCR

Amplification was performed in the exponential phase (20–28 cycles) to allow comparison among cDNAs synthesized from identical reactions, using specific primers (Table 1). Primers for *$p21^{waf1}$* and *GAPDH* genes were also applied, as described previously.[23,24]

Western Blot Assays

Total cellular proteins were prepared using RIPA buffer (50 mmol/l Tris-HCl (pH7.2), 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS). Nuclear and cytoplasmic fractions were also prepared using NE-PER nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL, USA). Western blot assays were performed as described previously.[19–21]

Chromatin Immunoprecipitation Assays

Chromatin immunoprecipitation (ChIP) analysis was performed using an EpiXplore ChIP assay kit (Clontech Lab, Mountain View, CA, USA). Briefly, after transient transfection of pcDNA mouse p65, cells were cross-linked with formaldehyde. Cell lysates were sonicated to share DNA to lengths between 200 and 1000 bp, and were then precipitated overnight using anti-NF-κB/p65 antibody or mouse IgG as a negative control, along with magnetic beads. After proteinase K digestion, DNAs were extracted and were analyzed by PCR. ChIP analysis was conducted with a reduction in the number of cycles from 30 to 25, using three specific primer sets.

Immunofluorescence

After cotransfection of pcDNA3.1-HA-β-catΔS45, pCMV-Flag-HIF-1α, or pCI-Flag-p300 into Ishikawa cells, the cells were incubated with primary antibodies to β-catenin, HIF-1α, p300, HA, and Flag. Fluorescein isothiocyanate- or rhodamine-labeled anti-mouse or rabbit IgG (Molecular Probe, Leiden, The Netherlands) were used as secondary antibodies, as described previously.[19–21]

Clinical Cases

Histological findings were reviewed for hysterectomy specimens of endometrioid type Em Cas in the case records of the Kitasato University Hospital during the period from 2000 to 2008, according to the criteria of the 2003 World Health Organization classification.[28] A total of 64 cases of Em Cas that included 42 of grade (G)1 or 2 and 22 of G3 were investigated. Of G1 tumors, 18 had squamous morules with high levels of nuclear β-catenin accumulation in the surrounding carcinomatous lesions.[19–21] In addition, 40 biopsy specimens of normal endometrial tissues that included 10 in the proliferative, 20 in secretory (10 early and 10 middle and late), and 10 in the menstrual phases were also selected. All tissues were routinely fixed in 10% formalin and processed for embedding in paraffin wax. Approval for

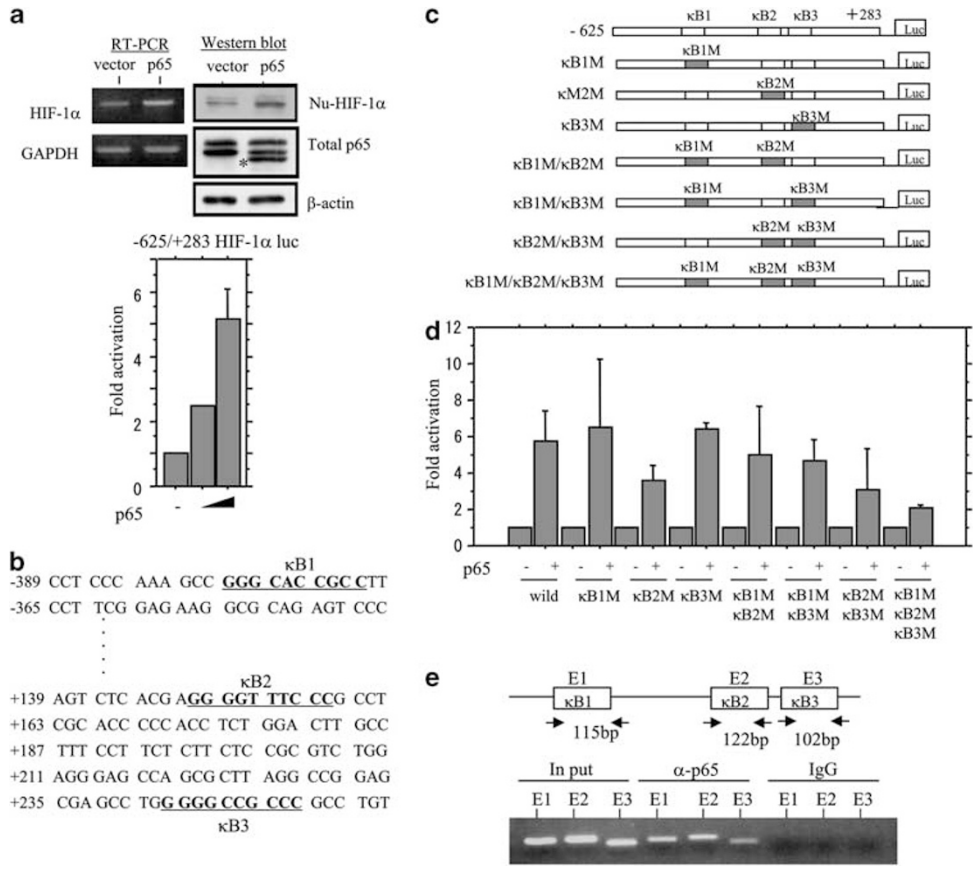

Figure 2 Transcriptional upregulation of hypoxia-inducible factor (HIF)-1α by nuclear factor-κB (NF-κB)/p65. (**a**) Upper: analysis of HIF-1α mRNA and protein expression levels. Total RNA or protein (Nu, nuclear fraction; Total, total fraction) was extracted from p65-transfected Ishikawa cells, and used in reverse-transcriptase PCR (RT-PCR) (left) and western blot (right) assays, respectively. Note the exogenous p65 (indicated by asterisk) in the middle panel of the western blot assay. Lower: Ishikawa cells were transfected with HIF-1α reporter constructs, together with p65. Relative activity was determined based on arbitrary light units of luciferase activity normalized to pRL-TK activity. The activities of the reporter plus the effector relative to that of the reporter plus empty vector are shown as means ± s.d. The experiment was performed in duplicate. (**b**) The *HIF-1α* promoter sequence containing three putative NF-κB-binding sites, including the κB1, κB2, and κB3 sites. (**c**) Various promoter constructs with mutations in the κB sites were used for evaluating transcriptional regulation of the *HIF-1α* promoter by p65. (**d**) Ishikawa cells were transfected with various mutant constructs of the *HIF-1α* promoter, along with p65. The experiment was performed in triplicate. (**e**) Chromatin immunoprecipitation (ChIP) assay data showing that overexpressed p65 binds to the *HIF-1α* promoter region containing κB1, κB2, and κB3 sites.

this study was provided by the Ethics Committee of the Kitasato University School of Medicine (B07-16).

Immunohistochemistry

Immunohistochemistry (IHC) was performed using a combination of the microwave heating and polymer immunocomplex (Envision, Dako) methods. For the evaluation of immunostaining, tumor lesions were subdivided into HIF-1α-positive and -negative carcinomatous components. Lesions with severe inflammation or necrotic features, probably associated with severe hypoxic effects, were excluded. Nuclei positive for HIF-1α, pp65, β-catenin, and Ki-67 were counted in at least 1000 tumors in each of 5 randomly selected fields for both components in all cases. Labeling indices (LIs) were then calculated as numbers per 100 cells, as described previously.[19–21] HIF-1α-positive carcinomatous lesions were defined when over 20% of cells were stained in each case. Scoring of nuclear immunoreactivity for HIF-1α in normal endometrial samples was also performed on the basis of the percentage of immunoreactive cells and the immunointensity by multiplying values for the two parameters, as described previously.[16–18]

In-Situ Hybridization Assays

The HIF-1α cDNA sequence (containing nucleotides 480 to 957; GenBank accession number BC012527) was subcloned into the pCRII vector (Invitrogen) by PCR-based strategy, using a specific primer set as described previously.[29] The riboprobes were generated by *in vitro* transcription using T7 or Sp6 RNA polymerases in the presence of digoxigenin-labeled UTP. *In-situ* hybridization (ISH) assays were carried out using the GenPoint Tyramide Signal Amplification System (Dako), according to the manufacturer's instructions.

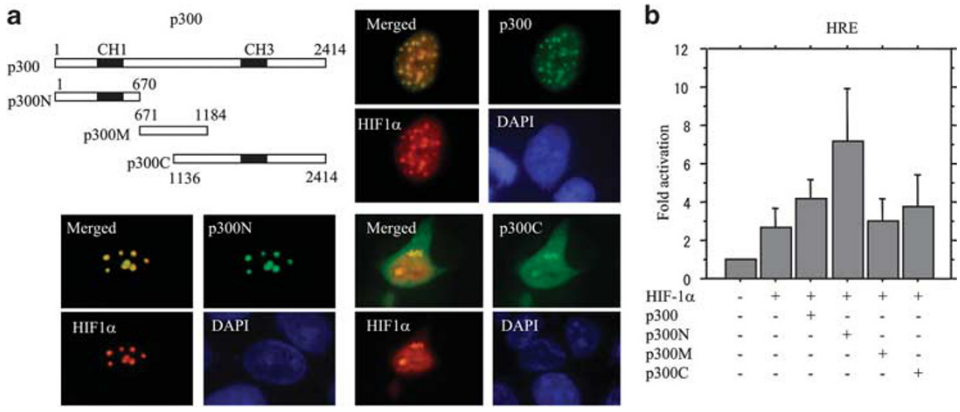

Figure 3 Interaction between hypoxia-inducible factor (HIF)-1α and p300. (**a**) Upper left: schematic representation of full-length p300 and the truncated forms (p300N, p300M, and p300C). Upper right: colocalization of exogenous HIF-1α and p300 constructs formed speckled nuclear bodies in Ishikawa cells. Lower: note the enlarged nuclear dots formed by colocalization of HIF-1α and p300N (left), in contrast to diffuse nuclear/cytoplasmic staining with a few nuclear aggregates by HIF-1α and p300C (right) in Ishikawa cells. (**b**) Ishikawa cells were transfected with HER reporter constructs, together with either p300 or its truncated forms. Relative activity was determined based on arbitrary light units of luciferase activity normalized to pRL-TK activity. The activities of the reporter plus the effector relative to that of the reporter plus empty vector are shown as means ± s.d. The experiment was performed in duplicate.

Statistics

Comparative data were analyzed using the Mann–Whitney *U*-test. The cut-off value for statistical significance was set as $P<0.05$.

RESULTS

Relationship Between Hypoxia and Cell Kinetics

The effects of $CoCl_2$ treatment on cell kinetics was examined, because it mimics the effects of hypoxia.[25–27,30] As shown in Figure 1a, long-term exposure of Ishikawa cells to $CoCl_2$ inhibited growth considerably as compared with controls, along with pronounced increase in HIF-1α protein expression but not mRNA levels. An increased $p27^{kip1}$ expression was also observed for both mRNA and protein levels but such expressions were relatively minor in cases of $p21^{waf1}$, and VHL. Hec251 cells showed similar responses to $CoCl_2$ (Supplementary Figure S1A).

Several *in vivo* studies showed an increase in HIF-1α mRNA levels in mice, rats, and ferrets under hypoxia.[31–33] To determine whether a similar response could be shown *in vitro*, Ishikawa cells were treated with short-term exposure to $CoCl_2$. As shown in Figure 1b, HIF-1α mRNA levels increased in response to hypoxia within 0.5 hh after $CoCl_2$ treatment, returning to basal levels after 2 h. In contrast, the protein levels rapidly increased within 0.5 h and remained stable for up to 6 h. An increase in nuclear NF-κB/p65 (p65) levels was also observed, peaking at 0.5 to 3 h, along with weak increases in the levels of cytoplasmic IκBα protein. In addition, pretreatment with the transcription inhibitor actinomycin D inhibited $CoCl_2$-mediated HIF-1α expression for both mRNA and protein levels (Figure 1c), strongly suggesting that the regulation occurs at transcriptional levels. Similar findings were also seen in Hec251 cells treated with short-term exposure to hydrogen peroxidase (Supplementary Figure S1B), which can act as a second messenger in the responding to both TNF and okadaic acid stimulation,[34] as well as Hec50 cells treated with $CoCl_2$ (Supplementary Figure S1C).

Transcriptional Relationship Between NF-κB/p65 and HIF-1α

Given that basal HIF-1α mRNA levels are controlled by several NF-κB subunits,[13–15] the role of NF-κB/p65 in regulating HIF-1α expression was studied. Transfection of p65 caused an increase in both HIF-1α mRNA and protein expression levels, in line with activation of the promoter, in a dose-dependent manner (Figure 2a).

On the basis of previous data that indicated a consensus binding sequence 5′-GGRNNYYCC-3′,[23,24] we noticed that a 900-bp fragment upstream of the translation start site in the *HIF-1α* gene was highly GC-rich and contained three potential binding sites for NF-κB (Figure 2b). Additional promoter constructs carrying four nucleotide alterations in the κB-binding sites, including κB1, κB2, and κB3 (Figure 2c), resulted in considerable reduction of response to p65 (Figure 2d). ChIP assays also revealed that transfected p65 recruited the *HIF-1α* promoter to the three κB-binding sites (Figure 2e), indicating that the three regions are required for the promoter to be stimulated by p65. In contrast, transfection of HIF-1α also exhibited an increase in basal- and p65-meditaed κB-luc reporter activity (Supplementary Figure S2A).

Relationships among HIF-1α, β-catenin, and p300

As the transcriptional activity of stabilized HIF-1α is exerted by recruiting the transcriptional co-activator and integrator p300/CBP,[35] the role in HIF-1α-dependent transcription was also examined. As shown in Figure 3a, overexpressing HIF-1α

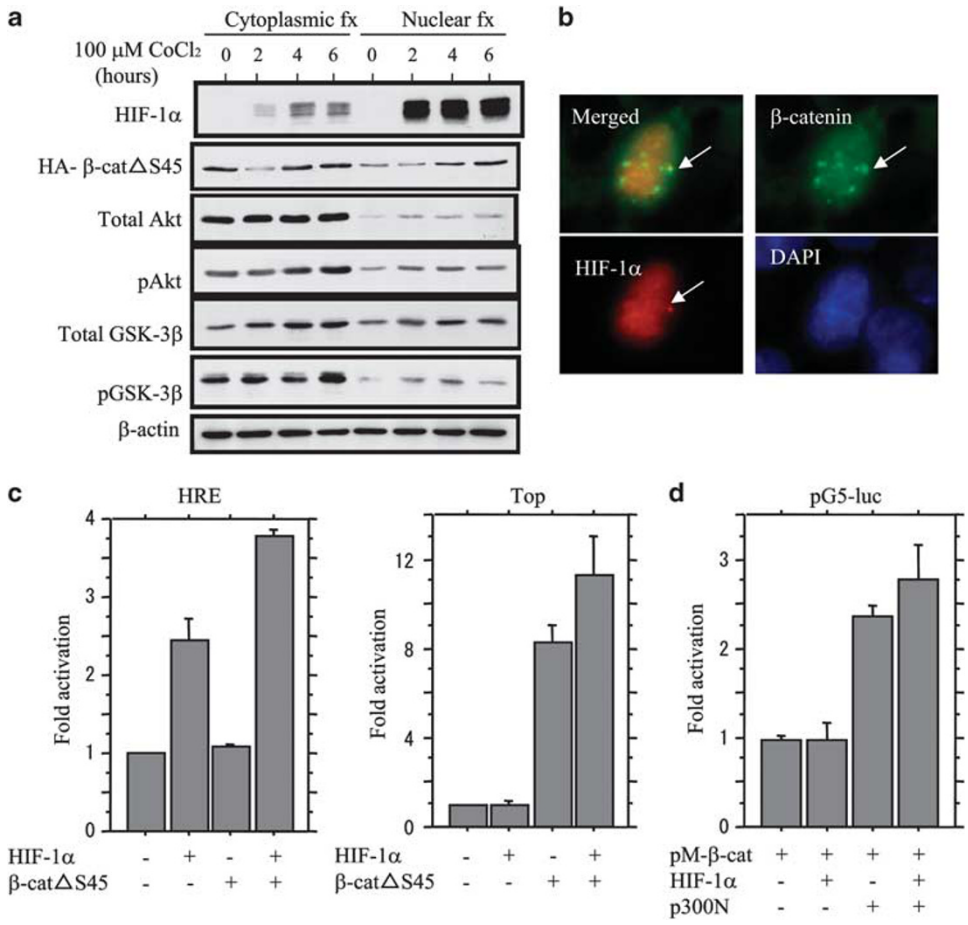

Figure 4 Hypoxia-inducible factor (HIF)-1α interacts indirectly with β-catenin through p300. (**a**) Western blot analysis of HIF-1α, HA-β- catΔS45, and total and phosphorylated (p) forms of both p65 and GSK-3β expression in cytoplasmic and nuclear fractions of Ishikawa cells stably overexpressing HA-β-catΔS45 for the time shown after 100 μM cobalt chloride ($CoCl_2$) exposure; fx, fraction. (**b**) Colocalization of exogenous HIF-1α and HA-β-catΔS45 formed a few nuclear aggregates in Ishikawa cells. (**c**) Ishikawa cells were transfected with either HER (left) or Top (right) reporter constructs, together with HIF-1α and/or β-catΔS45. Relative activity was determined based on arbitrary light units of luciferase activity normalized to pRL-TK activity. The activities of the reporter plus the effector relative to that of the reporter plus empty vector are shown as means ± s.d. The experiment was performed in duplicate. (**c**) Ishikawa cells were transfected with pG5-luc reporter constructs, together with pM-β-catΔS45, HIF-1α, and p300N. The experiment was performed in duplicate.

colocalized with the full length of p300 constructs, with a formation of nuclear speckles. Cotransfection with p300N-terminus also resulted in a formation of several enlarged nuclear dots, in contrast to diffuse nuclear/cytoplasmic staining with a few of nuclear aggregates by p300C but not p300M (data not shown). HRE reporter assays exhibited an eightfold increase in activity with a combination of HIF-1α and p300N, whereas such associations were relatively minor in cases of either p300M or p300C (Figure 3b), suggesting that HIF-1α interacts mainly with the N-terminus of p300.

Further studies were performed to determine whether subcellular localization of β-catenin is affected by exposure to hypoxia, as hypoxia is known to alter β-catenin/TCF-dependent transcriptional activity.[22,36] In Ishikawa cells stably overexpressing actively formed β-catenin, treatment with $CoCl_2$ resulted in an increased accumulation of β-catenin in the nuclear but not cytoplasmic fraction, as well as phosphorylated forms of both Akt and GSK-3β (Figure 4a). Colocalization of exogenous β-catenin and HIF-1α was also observed with a few small nuclear aggregates (Figure 4b). Moreover, cotransfection resulted in a slight enhancement of both HRE (HIF-1α)- and Top (β-catenin) reporter activities mediated by either HIF-1α or β-catenin (Figure 4c). Similar enhancement was also observed with the *Glut1* promoter (Supplementary Figure S2B), a target gene for HIF-1α.[37] In contrast, one hybrid assay revealed little activity of the pG5-luc reporter by cotransfection of the DNA-BD-fused full-length β-catenin fragment (pM-β-cat) and HIF-1α, in contrast to p300N (Figure 4d), indicating that the associations may be indirect.

IHC and ISH Findings in Normal and Malignant Endometrium

Examples of the IHC findings for HIF-1α in the normal endometrium during the menstrual cycle are illustrated in Figure 5a. A distinct nuclear immunoreactivity for HIF-1α

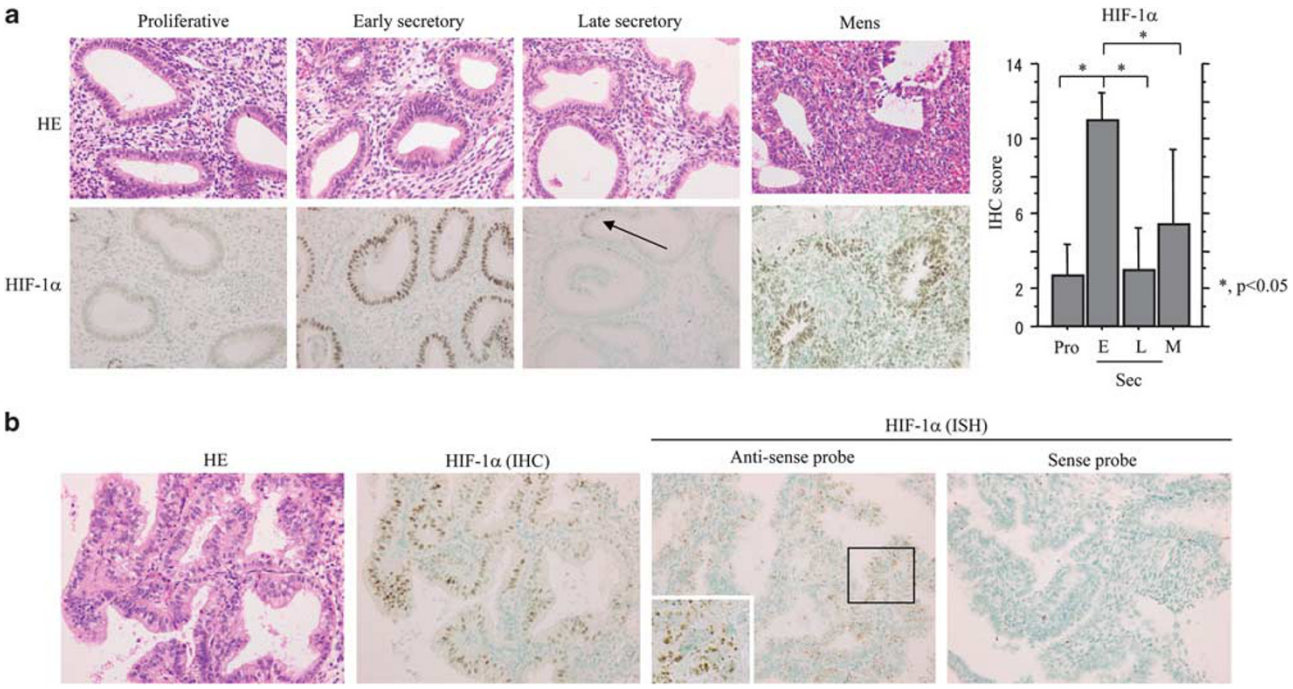

Figure 5 Immunohistochemistry (IHC) and *in-situ* hybridization (ISH) findings of hypoxia-inducible factor (HIF)-1α in normal and malignant endometrial tissues. (**a**) Left: staining with hematoxylin and eosin (HE; upper panels) and by IHC for HIF-1α in the proliferative, the early and late secretory, and the menstrual (Mens) stages (lower panels). Note the strong nuclear staining for HIF-1α in early secretory epithelial components in contrast to the weak immunoreaction in proliferative and late secretory stages (indicated by arrows). Original magnification, × 200. Right: IHC scores for HIF-1α in the proliferative (Pro), early (Sec-E) and late secretory (Sec-L), and menstrual (M) stages. The data shown are means ± s.d. (**b**) Serial sections of endometrial carcinoma (Em Ca) tissue. Staining with hematoxylin and eosin (HE) and by IHC and ISH for HIF-1α. Note the distinct nuclear staining and mRNA signals (with the enclosed box magnified in the inset) of HIF-1α. Original magnification, × 200 and × 400 (inset). *P-value.

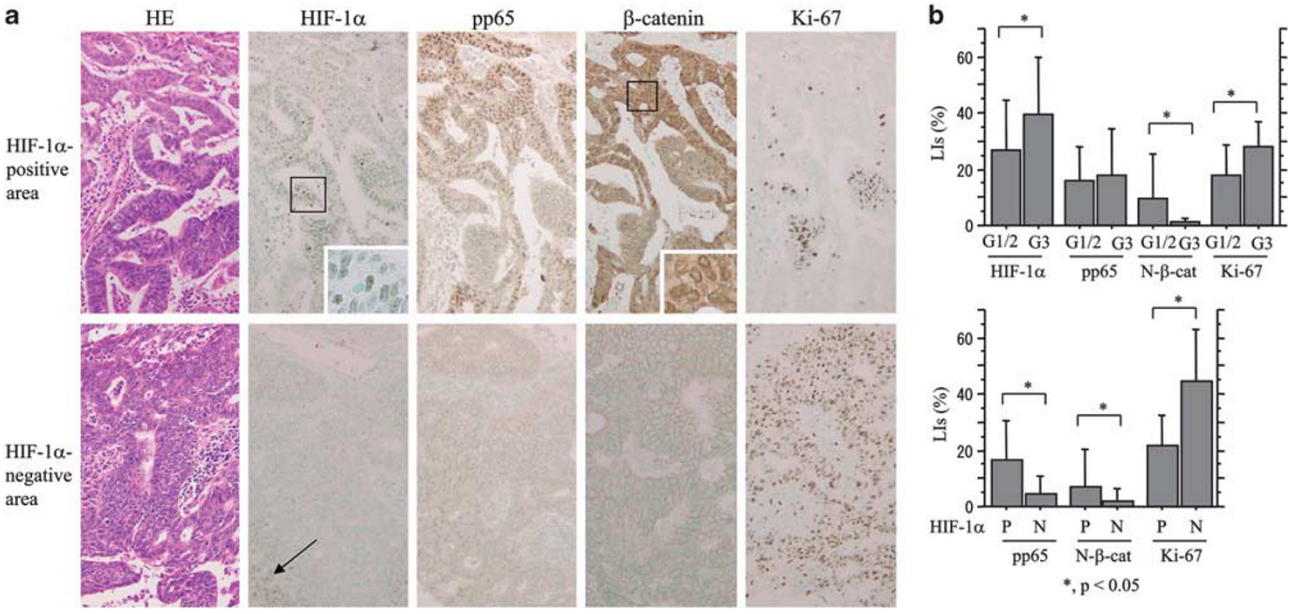

Figure 6 Immunohistochemistry (IHC) findings in serial sections of endometrial carcinomas (Em Cas). (**a**) Staining with hematoxylin and eosin (HE) and by IHC for hypoxia-inducible factor (HIF)-1α (with the enclosed box magnified in the inset), pp65, β-catenin (with the enclosed box magnified in the inset), and Ki-67 in HIF-1α-positive (upper) and -negative components (lower) of grade 1/2 Em Cas. Original magnification, × 100 and × 400 (inset). (**b**) Upper: nuclear (N) labeling indice (LI) values for HIF-1α, pp65, β-catenin (cat), and Ki-67 in grade (G) 1/2 and 3 Em Cas. The data shown are means ± s.d. Lower: nuclear (N) LI values for pp65, β-catenin (cat), and Ki-67 in HIF-1α-positive (P) and -negative (N) carcinomatous lesions of Em Cas. The data shown are means ± s.d. *P-value.

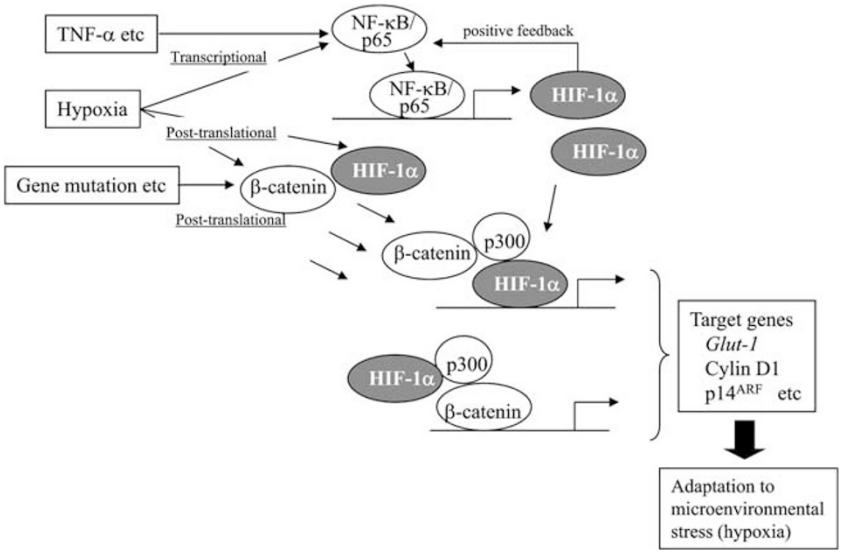

Figure 7 Schematic representation of associations among hypoxia-inducible factor (HIF)-1α, nuclear factor-κB (NF-κB), and β-catenin in response to microenvironment factors including hypoxia in endometrial carcinoma (Em Ca) cells.

was observed generally in epithelial but not stromal components, with the exception of those in menstrual phase. The average IHC scores for HIF-1α were significantly higher in the early secretory than the other menstrual stages.

To examine the relationship of HIF-1α expression with mRNA and protein levels, ISH assays were carried out using 10 Em Ca cases. In carcinoma components without necrotic lesions, positive signals for HIF-1α mRNA appeared to be consistent with high levels of the nuclear immunopositivity in most cases (Figure 5b).

Examples of the IHC findings for HIF-1α, pp65, β-catenin, and Ki-67 are shown in Figure 6a. Immunoreactivity for HIF-1α appeared to overlap with nuclear staining for pp65 and β-catenin but not Ki-67. As shown in Figure 6b, average LI values of HIF-1α, as well as Ki-67, were significantly higher in G3 than G1/2 tumors, in contrast to nuclear β-catenin and pp65. Moreover, significant differences in LIs of pp65, β-catenin, and Ki-67 were observed between HIF-1α-positive and -negative carcinomatous lesions.

DISCUSSION

Evidence has been provided that HIF-1α expression is regulated not only by post-translational modification but also involved transcriptional control by NF-κB/p65, from the following findings. First, short-term treatment of cells with $CoCl_2$ and H_2O_2 resulted in a rapid and transient increase in HIF-1α mRNA expression, along with stabilization of nuclear p65. At the same time, pretreatment with the transcriptional inhibitor actinomycin D resulted in suppression of the mRNA and protein expression induced by $CoCl_2$ treatment. Second, cells transiently overexpressing p65 exhibited upregulation of HIF-1α mRNA and protein expression through activation of the promoter, in line with the data showing significantly higher pp65 LI values in HIF-1α-positive as compared with HIF-1α-negative components in Em Cas. Third, p65 was capable of binding directly to the three specific regulatory sequences, including nucleotides −377/−368, +149/+158, and +244/+253, within the promoter, as evidenced by inhibition of promoter activity by mutations in the three sequences. In contrast, previous studies demonstrated that activated NF-κB was bound to the putative responsive site at −197/−188 of the promoter in human pulmonary artery smooth muscle cells.[13,38] Other cell type-specific transcriptional factors may also contribute to the promoter-specific mechanism of action.

In contrast to transcriptional upregulation of HIF-1α by NF-κB, overexpression of HIF-1α also caused an increase in NF-κB-mediated transcription, indicating the existence of a positive feedback loop system. This conclusion is supported by our results of high levels of pp65 expression in HIF-1α-positive components of Em Cas. The significantly lower cell proliferation in HIF-1α-positive carcinomatous lesions may be due to the relatively low-oxygenated status, as hypoxia caused cell cycle arrest through a mechanism that was either dependent (p53, $p21^{waf1}$, and bcl-2) or independent (GADD153) of HIF-1α.[39] In fact, long-term treatment of Ishikawa cells with $CoCl_2$ resulted in a significant decrease in growth, leading to upregulation of $p27^{kip1}$.

A rapidly growing body of evidence indicates that hypoxia triggers a functional switch in β-catenin signaling, as well as PI3K/Akt.[22,35,40–42] In this study, $CoCl_2$-meditated hypoxia caused considerable accumulation of nuclear β-catenin, probably due to inactivation of the Akt/GSK-3β-mediated degradation system. This is consistent with the results of significantly higher levels of nuclear β-catenin in HIF-1α-positive lesions of Em Cas. However, the association appeared to be indirect, on the basis of our findings, in one hybrid assay. Given the evidence of a direct association

between p300 and either HIF-1α or β-catenin,[43] it is likely that the co-activator may be essential for cross-talk between the two signaling components in Em Ca cells.

Another finding of interest in this study was the transient upregulation of HIF-1α expression that was observed in normal endometrial glandular but not stromal components in early secretory stages, despite relatively well-oxygenated tissues. A similar finding was also observed in a case of Sox7, which may be important for changes in cell kinetics from proliferative to secretory stages in the normal endometrium.[44] Interestingly, HIF-1α protein has been shown to be upregulated under normoxic condition in response to growth factors, hormones, coagulation factors, cytokines, and vasoactive peptide,[45–48] allowing us to speculate that other factors, in particular estrogen, may serve as positive regulators for HIF-1α expression during the menstrual cycle, independent of O_2 tension. In contrast, the relatively higher HIF-1α expression in both glandular and stromal components in menstrual stages may be simply due to hypoxic effects.

Our observations suggest a model for the regulation and function of HIF-1α in Em Ca cells (Figure 7). Activation of the NF-κB pathway due to a variety of stimuli, such as hypoxic condition and several cytokines, causes transactivation of the *HIF-1α* gene, while HIF-1α can enhance NF-κB-mediated transcriptional activity, through a positive feedback loop system. Overexpression of HIF-1α is also associated indirectly with excess β-catenin, probably due to the gene mutations and hypoxic exposure, by interaction with p300, and this in turn leads to transactivation of their target genes, resulting in tumor adaptation to microenvironmental hypoxia in Em Ca cells.

In conclusion, our results suggest that associations with HIF-1α and the NF-κB pathway, as well as β-catenin/p300 complexes, may participate in modulating changes in tumor kinetics in response to hypoxic stress in Em Cas.

Supplementary Information accompanies the paper on the Laboratory Investigation website (http://www.laboratoryinvestigation.org)

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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